Use of a mixture of glucose and methanol as substrates for the production of recombinant trypsinogen in continuous cultures with *Pichia pastoris* Mut+  

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**A B S T R A C T**  
Pure methanol, which is required as an inducer of the AOX1 promoter and a carbon/energy source in processes for recombinant protein production by *Pichia pastoris*, is impracticable and therefore generally undesirable. As an alternative, a procedure using double carbon substrate was examined (11.7 g carbon L\(^{-1}\), 60%/40% carbon from glucose/methanol). The effects on methanol metabolism, extracellular formation of porcine trypsinogen, biomass growth and cell viability were analyzed. In contrast to batch cultures, where the glucose and methanol were utilized sequentially, in carbon/energy-limited continuous cultures (operated between dilution rates 0.03 and 0.20 h\(^{-1}\) the repressive effect of glucose on methanol utilization was eliminated up to 0.15 h\(^{-1}\) (ca. 130% of \(\mu_{\text{max}}\) with methanol). With the mixture, the yield of biomass (1.54 ± 0.12) g\(\text{CDW}\) L\(^{-1}\) was found to be 1.4 times larger than the yield with methanol alone. Despite the current widespread view that glucose has a repressive effect on the AOX1 promoter, the product was synthesized over the entire range of dilution rates, with maximum productivities of (0.70 ± 0.12) mg\(\text{CDW}\) h\(^{-1}\) at 0.07 h\(^{-1}\). Thus, glucose was shown to be a feasible partial substitute for methanol in recombinant protein production by *Pichia pastoris* Mut+ strain while enhancing process productivity.

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1. Introduction  
The *Pichia pastoris* yeast system is much sought after for the industrial manufacturing of recombinant proteins and is also an interesting target for further improvement at genetic level (Cereghino and Cregg, 2000; Kovar et al., 2010). Despite the availability of other promoters (Blank and Kuepfer, 2010; Hartner et al., 2008; Stadlmayr et al., 2010; Waterham et al., 1997), the most widespread technology currently used in the production of recombinant proteins with *P. pastoris* is still the expression of a targeted heterologous gene under the tightly controlled AOX1 promoter (Celik et al., 2010; Cregg et al., 1987; Jungo et al., 2007b; Sola et al., 2007; Tschopp et al., 1987). This promoter utilizes the function of the first step in methanol metabolism, which is catalyzed by alcohol oxidase I (AOX1). Advantageously, it is one of the strongest naturally occurring promoters, and has generally enabled exceptionally high transcriptional levels to be achieved.

The common procedure for recombinant protein production with *P. pastoris* (Mut+) typically uses only methanol that simultaneously acts as the inducer of the AOX1 promoter and as the single source of carbon and energy (Invitrogen, 2002; Tolner et al., 2006). Though demanding, the high oxygen requirements and the associated heat evolution, in addition to the potential flammability and explosiveness of methanol, pose solvable challenges to large scale process development (e.g. Inan and Meagher, 2001; Jungo et al., 2007a; Khatri and Hoffmann, 2006). Moreover, addition of methanol needs to be controlled by an advanced dosing system to prevent (1) its accumulation in the culture broth up to undesired levels as well as (2) the subsequent increase in formaldehyde (the first metabolite of methanol catabolism) concentration above its toxic limits. In fedbatch or continuous culture methanol is added under carbon-limited growth conditions (Hyka et al., 2010) or to maintain a constant methanol concentration in culture, controlled using a methanol sensor (Hellwig et al., 2001; Zhang et al., 2004). The simultaneously formed formaldehyde and hydrogen peroxide can, at a certain concentration, cause oxidative stress and compromise the cells’ viability (Higgins and Cregg, 1998; Xiao et al., 2006; Yan et al., 2009).

A decrease in the number of actively producing cells not only reduces the efficiency of the process, but lysed cells may also release undesirable host cell proteins and/or proteases that accelerate product degradation (Cregg et al., 2000; Hyka et al., 2010; Jahic et al., 2003b).
In principle, the obstacles related to the use of methanol can be overcome by technical bioreactor improvements, by using a different type of promoter (Blank and Kuepfer, 2010; Hartner et al., 2008; Stadlmayr et al., 2010; Waterham et al., 1997), or by replacing part of the methanol with a complementary carbon substrate which does not repress the AOX1 promoter (Files et al., 2001; Hellwig et al., 2001; Jungo et al., 2007a; Katakura et al., 1998; Zhang et al., 2003). Although several strategies to supply various carbon sources (e.g. sorbitol, glycerol, lactate or acetic acid) together with methanol have already been described (Celik et al., 2010; Inan and Meagher, 2001; Sola et al., 2007; Wei et al., 2008; Xie et al., 2005), glucose has only been sporadically mentioned as a suitable substrate for the cultivation of P. pastoris Mut+ strains (Brierley et al., 1990; Hang et al., 2008; Okabayashi et al., 1997). Typically, the supposed repressive effect of an excess of glucose on methanol metabolism was highlighted. However, using glucose together with methanol for cultivation of C4-utilizing microorganisms is not a new idea, the growth of other non-recombinant methylotrophic yeasts in carbon-limited cultures containing mixtures of glucose and methanol was examined 30 years ago (Egli et al., 1982a,b). It was demonstrated that depending on the enzyme and the organism, 50–90% of glucose in the mixture allows the enzymes in the methanol utilization pathway to function at their highest levels of activity (Egli and Mason, 1991; Egli and Harder, 1984). Moreover, using glucose as a substitute for part of the methanol enabled the yeast to be grown at dilution rates significantly exceeding values achieved with pure methanol (Egli et al., 1986).

Although the repression/derepression mechanism of the AOX1 promoter in P. pastoris is similar to that regulating methanol (alcohol) oxidase in H. polymorpha (Egli et al., 1986; Giuseppin et al., 1988; Giuseppin, 1989), unlike H. polymorpha, a certain portion of the promoter is essential in P. pastoris for the production of recombinant protein (Tschopp et al., 1987). Thus, specific AOX-activities reported for chemostat cultures with glycerol alone amounted to only 1–5% of the activities detected in cultures with methanol (Jungo et al., 2006).

Based on data published for non-recombinant Hansenula (Egli et al., 1986) and Kloeckera (Egli et al., 1983) and other preliminary experiments (not published), a mixture consisting of 40% carbon from methanol and of 60% carbon from glucose was examined as an alternate strategy for the production of recombinant protein in P. pastoris Mut+ strains. The feasibility of using a mixed substrate was tested on the production of an extracellularly secreted model product, porcine trypsinogen, cloned under the control of the AOX1 promoter. Trypsinogen, a zymogen of the widely applied serine protease trypsin (24.4 kDa, EC 3.4.21.4), is currently gained through extraction from animal pancreas tissue. Due to increasing use of trypsin in clinical applications, there is growing interest in the microbial manufacturing of a high quality and thus regulatory approvable product. When secreted by yeast cells, it is feasible to produce inactive trypsinogen in a pure form, rather than trypsin or a mixture of trypsin and trypsinogen. When (free) trypsin is present in the culture supernatant, degradation of trypsinogen is accelerated due to its proteolytic activity; this leads to further autocatalytic degradation at pH values higher than 4. In addition, trypsin is not desirable since it causes physiological stress on microbial cells, which is typically followed by cell lysis (Hanquier et al., 2003, 2000; Hyka et al., 2010; Keil et al., 1959).

The current physiological study was carried out in continuous cultures of P. pastoris Mut+ grown over a wide range of dilution rates (between 0.03 and 0.20 h−1) to analyze (i) the effect of a mixture of glucose and methanol on methanol metabolism, (ii) the formation of extracellular recombinant product, and (iii) biomass growth and cell viability.

2. Materials and methods

2.1. Microorganism

A strain producing porcine trypsinogen and containing the synthetic gene of porcine trypsinogen (GeneArt AG, Regensburg, Germany), was used, that is, the P. pastoris X33 strain using the AOX1 promoter transformed with a plasmid based on pPIC3KαA (Invitrogen, Carlsbad, CA). Codon usage was optimized for P. pastoris. The selection of transformant was based on ZeocinTM (Invitrogen, Carlsbad, CA) resistance of the transformation vector. The strain showed a Mut+ (methanol utilization-positive) phenotype and contained the codon-optimized mating α-factor leader signal sequence of S. cerevisiae for extracellular protein secretion. The stock cultures were conserved in 24% glycerol at −80 °C.

2.2. Culture media

The buffered glycerol complex medium (BMGY) (Invitrogen, 2002) used for the precultures contained 10 g glycerol, 10 g yeast extract, 20 g peptone, 100 ml of 1 M potassium phosphate buffer (pH 6.0), 13.4 g yeast nitrogen base without amino acids, 0.4 mg biotin and 0.1 g of zeocin per liter. Defined minimal medium contained (11.7 ± 1.1) g of carbon source (i.e. only glucose for the starting batch cultures, and only glucose, only methanol, or a mixture of glucose and methanol (60:40) for continuous cultures), 0.17 g CaSO4·2H2O, 2.86 g K2SO4, 0.64 g KOH, 2.3 g MgSO4·7H2O, 0.2 g EDTA, 7.23 g H3PO4, 9.55 g NH4Cl, 0.1 ml polypropylene glycol (PPG) per liter (all of which were autoclaved), 4.35 ml filter sterilized PTM1 solution, and 0.87 mg biotin per liter, which were added separately. The PTM1 stock solution (Invitrogen, 2002) contained 5.0 ml 96% H2SO4, 3.4 g CuSO4·5H2O, 0.08 g NaI, 3.0 g MnSO4·H2O, 0.2 g Na2MoO4·2H2O, 0.02 g H3BO3, 0.92 g CoCl2·6H2O, 20.0 g ZnCl2, and 65.0 g FeSO4·7H2O per liter. Before inoculation, the original pH of the medium (2.5) was adjusted to 5.9 using 25% NH4OH. The batch experiment to study diauxic growth was performed with a mixture of 2.8 g glucose and 4.5 g methanol per liter. The composition of the carbon mixture (40% C6 and 60% C4) was chosen in accordance with published data (Brierley et al., 1990; Egli et al., 1982b; Egli and Mason, 1991; Egli and Harder, 1984) and preliminary experiments. Comprehensive studies on the utilization of methanol and glucose with different ratios of both substrates by methylotrophic yeast show that, in a glucose/methanol mixture, approximately 10–50% methanol is sufficient to fully induce any enzyme of methanol catabolism, and that alcohol oxidase is not repressed by glucose added under carbon-limited growth conditions.

A glycerol stock (1 ml) of strain was thawed and used to inoculate a shake flask with 150 ml of BMGY medium. This first seed culture was grown over 24 h at 28 °C and 150 rpm, during which the optical density at 600 nm (OD600nm) increased from 0.1 to 0.57. From this cell suspension, 15 ml was transferred to 135 ml of BMGY medium and grown for 12 h at 30 °C and 150 rpm, during which the OD600nm increased from approximately 6 to 30. The second preculture was aseptically transferred to the bioreactor to achieve a 10% inoculation ratio (final OD600nm, ca. 3).

2.3. Culture conditions

All cultivation processes were carried out in a 2-l bioreactor (BIOSTAT, B. Braun Biotech International, Germany), with the working volume during continuous operation kept constant at 1.5 l by removal of effluent from the bioreactor using a peristaltic pump (Verderlab Pericor, Verder, Austria). Processes were performed at a constant temperature of 30 °C, with an airflow of 2.25 l min−1, and 800 rpm agitator speed. Ammonium hydroxide (25%) and...
phosphoric acid (8.5%) were used to maintain the pH at a constant value of 5.9. The process was initiated with a batch culture of 1.5-1 working volume, containing 33 g of glucose monohydrate (11.7 g of carbon) per liter. The relative partial pressure of oxygen (pO$_2$) in the medium was not regulated, which resulted in a continuous decrease in the pO$_2$ value during batch culture, but did not decrease below 20% over the whole cultivation process. Between 12 and 14 h, the pO$_2$ value increased rapidly, at which point continuous culture was initiated. During continuous operation, the mineral medium with methanol or glucose alone as well as the mixture of glucose and methanol examined was fed into the bioreactor using a programmable peristaltic pump (Watson Marlow 505S, Watson Marlow Limited, UK), the inflow of media being set to reach the required dilution rate D (defined as volumetric flow rate per working volume). Samples were withdrawn regularly during both transient and steady states. Steady-state data were typically collected from multiple samples taken after 3–5 residence times when the concentrations of biomass, substrates and pO$_2$ reached constant values.

2.4. Substrate analyses

Concentrations of glucose and methanol were determined in culture supernatants by HPLC analysis (Agilent, Spain) using Polymer H$^+$ column (Watrex Prague, Czech Republic) at 80 °C and a refractive index detector (Agilent, Spain). A mobile phase of 1 mM H$_2$SO$_4$ in demineralized water was applied at 0.5 ml min$^{-1}$ flow rate, with an injection volume of 20 μl. The limit of detection was 0.10 g l$^{-1}$ for methanol and 0.03 g l$^{-1}$ for glucose.

2.5. Concentration of biomass

The concentration of biomass was determined gravimetrically as cell dry weight, the cells generally being dried at 105 °C after centrifugation and washing.

2.6. Recombinant protein analyses (from both enzyme activity and HPLC)

Trypsinogen concentration was determined after activation with endorokinase in 0.15 mol l$^{-1}$ TEA buffer pH 8.2 for 24 h at 4 °C. One unit of trypsin hydrolyzes 1 μmol of chromogenic substrate Nα–Benzoyl–arginine-p–nitroanilid (βApNA) per minute at 25 °C at pH 8.2. (Shodex, Japan) was applied at 405 nm with UV-Visible Spectrophotometer Cary 50 Bio (Varian, Australia). The detection limit was 2.50 μg ml$^{-1}$.

HPLC (Agilent Series 1200, Spain) was based on the principle of hydrophobic interaction, was used for the quantification of recombinant α–trypsin, β–trypsin and trypsinogen with HIC PH-814 column (Shodex, Japan) at 25 °C. Mobile phases A containing 1.8 M (NH$_4$)$_2$SO$_4$ in buffer B, pH 3.0, and B containing 0.1 M PBS (KH$_2$PO$_4$+H$_2$PO$_4$), pH 3.0 were applied at a flow rate of 0.7 ml min$^{-1}$ using two linear gradients: for the period 0–60 min from 100% A to 100% B followed by 60–70 min from 100% B to 100% A and 70–90 min with 100% A for column regeneration. A multi–wave spectrophotometric detector (DE43603372, Agilent, Spain) set at 210 and 280 nm was used for quantification. The detection limit was 3.0 mg l$^{-1}$.

2.7. Viability analysis

A flow cytometer device (PAS III Partec, Partec, Germany) equipped with an argon-ion laser was employed for fluorescence measurement. Propidium iodide (PI, Sigma–Aldrich, USA) and bis-(1,3-dibutylbarbituric acid) trimethine oxonol (BOX, Sigma–Aldrich, USA) were used to assess cell membrane integrity and membrane polarity respectively. The staining procedures were optimized for Pichia conditions as described in the literature (Hyka et al., 2005). Prior to analysis, cells were centrifuged (1680 × g, 3 min), resuspended in filtered (0.22 μm, Sterifil™, Millipore, USA) phosphate-buffered saline (PBS) to optical density OD$_{500}$nm of 0.5, and incubated with 10 μg ml$^{-1}$ of PI for 5 min or 2 g ml$^{-1}$ of BOX for 20 min.

2.8. Data analysis

Specific rates of glucose, methanol and (total) carbon utilization/uptake were calculated according to the following equation:

\[ \text{q}_c = D \cdot \frac{(\text{c}_r - \text{c}_{i,in})}{\tau} \]  

(1)

where D is a dilution rate, c$_{i,in}$ and c$_r$ are concentrations of substrate in the inflowing medium and in the bioreactor in a steady state, respectively, and τ is the concentration of biomass in a steady state.

Wash-out experiments were accomplished with a dilution rate approximately three times higher than the values determined for maximum specific growth rates, above which the cells started to wash out from the bioreactor.

Maximum specific growth rates and maximum yields were calculated by performing linear regression using the following equations (i.e. for the natural logarithm of biomass versus time, and for the biomass production rate versus the carbon utilization rate respectively):

\[ \ln x = \ln x_0 + (\mu_{max} - D) \cdot t \]  

(2)

\[ Y_{x/c} = \frac{r_x}{r_C} \]  

(3)

where x is the concentration of biomass, D the dilution rate, $\mu_{max}$ the maximum specific growth rate, t time, $Y_{x/c}$ the yield, and $r_x$ and $r_C$ are the (volumetric) rates of biomass growth and carbon consumption respectively.

Specific production rates and volumetric productivity of extracellularly produced trypsinogen were calculated according to Eqs. (4) and (5) respectively:

\[ q_p = D \cdot \frac{\text{p}}{\tau} \]  

(4)

\[ r_p = D \cdot \frac{\text{p}}{\tau} \]  

(5)

where D is a dilution rate, and $\text{p}$ and $\tau$ are concentrations of product and biomass in a steady state.

Theoretical wash-in curves of methanol and wash-out curves of trypsinogen were calculated according to Eqs. (6) and (7) respectively:

\[ c_i(t) = c_{i,in} \cdot (1 - e^{-D \cdot t}) \]  

(6)

\[ p_i(t) = p_{i,0} \cdot e^{-D \cdot t} \]  

(7)

where $p_{i,0}$ is the concentration of trypsinogen in the bioreactor at the start of theoretical wash-out.

The number of residence times (RT) and the number of generations (NG) were calculated according to Eqs. (8) and (9) respectively:

\[ RT = D \cdot t \]  

(8)

\[ NG = \frac{RT}{\ln 2} \]  

(9)

3. Results

The feasibility of substituting glucose for part of the methanol substrate was tested on the production of recombinant trypsinogen by P. pastoris Mut+ grown in continuous cultures at dilution rates ranging between 0.03 and 0.20 h$^{-1}$. The culture behavior at
dilution rates higher than 0.20 h\(^{-1}\) was not examined due to the high accumulation of methanol, even though \(P.\) \textit{pastoris} is able to utilize glucose at such high dilution rates. Continuous cultures with only methanol or glucose as well as with a mixture of glucose and methanol (60:40% carbon) were examined, where the sum of carbon originating from glucose and from methanol was always kept at (11.7 ± 1.1) g\(\text{carbon}\) l\(^{-1}\).

### 3.1. Behavior in batch culture

In batch experiments a glucose and methanol mixture was supplied only at the time of inoculation. Glucose was consumed preferentially and, until its concentration reached zero, the enzymes of methanol utilization were not synthesized and recombinant protein was not produced (Fig. 1, phase A). In a subsequent adaptation phase, where the pool of enzymes required for methanol utilization was activated, the utilization of methanol commenced and formate (an intermediate of methanol metabolism) was produced (Fig. 1, phase B). The recombinant protein was synthesized at a later stage, simultaneously with methanol utilization (Fig. 1, phase C). A stationary phase of growth, with subsequent product degradation, occurred after the carbon/energy source was exhausted (Fig. 1, phase D).

### 3.2. Kinetics and stoichiometry of growth and substrate utilization in continuous cultures

Unlike batch cultures, in which diauxic (sequential) growth was caused by repression of methanol utilization by glucose (Fig. 1), in continuous cultures both co-substrates, that is, methanol and glucose, were supplied and utilized simultaneously (Fig. 3). The maximum stoichiometric and kinetic parameters of \(P.\) \textit{pastoris} cultures grown with individual substrates were determined (in triplicate) in wash-out experiments in which glucose or methanol were used as the sole substrate. The cells were grown in excess concentrations of each single substrate while exposed to dilution rates exceeding the particular maximum specific growth rate \((\mu_{\text{max}})\). Maximum specific growth rates of \((0.280 ± 0.012)\) h\(^{-1}\) and \((0.116 ± 0.002)\) h\(^{-1}\) for glucose and methanol respectively were determined by linear regression (Eq. (1)).

For the substrate mixture studied, the maximum specific growth rate was determined in experiments at the transient dilution rate \((D_0, 0.15\) h\(^{-1}\)). \(D_0\) is a value equal to the highest dilution rate at which both substrates were still completely utilized. Replacing 60% of the carbon from methanol by glucose thus enabled the critical dilution rate to be increased 1.3 times compared to a value of \(\mu_{\text{max.meth}}\) achieved with methanol alone (Table 1). The yield coefficients of biomass produced per carbon consumed for the individual substrates were determined from steady-state concentrations of biomass and the particular substrate (Eq. (2)). The yield using the mixture was determined at \(D < D_0\) by linear regression from the specific consumption rate of carbon \((\dot{q}_c)\) versus the dilution rate (Fig. 2, dashed line). The yield from a culture grown with a mixture of methanol and glucose was 1.4 times higher.

#### Table 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(\mu_{\text{max}}) (h(^{-1}))</th>
<th>(Y_{\text{X/carbon}}) (g\text{CDW g}^{-1}\text{carbon})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol(^a)</td>
<td>0.12 ± 0.01</td>
<td>1.08 ± 0.01</td>
</tr>
<tr>
<td>Glucose(^b)</td>
<td>0.28 ± 0.01</td>
<td>1.80 ± 0.07</td>
</tr>
<tr>
<td>Glucose and methanol(^c)</td>
<td>0.15 ± 0.01</td>
<td>1.54 ± 0.12</td>
</tr>
</tbody>
</table>

\(^a\) Determined from wash-out experiments.

\(^b\) Determined from steady-states at different dilution rates, where 0.15 h\(^{-1}\) corresponds to the transition rate \(D_0\), below which both substrates were utilized simultaneously and completely (Fig. 2).

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**Fig. 1.** Diauxic growth of \(P.\) \textit{pastoris} Mut\(^+\) strain with glucose and methanol in batch culture. Symbols – data from offline measurements; dashed lines – simulations using an exponential growth model. The process phases with respect to substrate utilization and tryptophan production are as follows: A – utilization of glucose and stagnation of methanol concentration at initial values, \(\mu = (0.26 ± 0.04)\) h\(^{-1}\), \(Y_{\text{X/carbon}} = (1.78 ± 0.17)\) g\text{CDW g}^{-1}\); B – activation of methanol metabolism and production of formiate (intermediate of methanol metabolism).  C – utilization of methanol and production of recombinant protein, \(\mu = (0.08 ± 0.02)\) h\(^{-1}\), \(Y_{\text{X/carbon}} = 0.94\) g\text{CDW g}^{-1}\); D – stationary phase. Standard deviations from triplicate measurements were below 3%.

**Fig. 2.** Specific conversion rates as a function of the dilution rate. The distinct process phases with respect to substrate utilization and tryptophan production in continuous cultures grown with a mixture of glucose and methanol are as follows: I – recombinant protein production stable with time, with simultaneous utilization of glucose and methanol; II – unstable (decreasing with time) production of recombinant protein, but with simultaneous and complete utilization of glucose and methanol; \(\mu_{\text{max.meth}}\) is the critical specific growth rate at which biomass wash-out begins if cells are grown with methanol alone and \(D_0\) the transition rate up to which both substrates in the mixture are simultaneously and completely utilized; III – unstable production of recombinant protein, which decreases with time and methanol accumulation, with complete utilization of glucose. Specific consumption rates of carbon were determined for glucose \((\dot{q}_c)\)/methanol \((\dot{q}_c)\) and the mixture of both substrates \((\dot{q}_c)\). Production rates are shown as squares, calculated using Eq. (4) from steady-state data \((\dot{q}_c)\) (closed) and, for cultures with unstable productivity, from arbitrary product measurements once 4 generations had been completed \((\dot{q}_c)\) (open). The dashed line represents the reciprocal graphical interpretation of yield of biomass per gram of consumed carbon being \((1.54 ± 0.12)\) g\text{CDW g}^{-1}\text{carbon} (Eq. (1)).
at (1.54 ± 0.12) g DW g\text{-1} carbon, than yields from cultures containing methanol alone (Table 1).

Both the specific rates of glucose and methanol uptake (Eq. (3)) were affected by the dilution rate, which reflects the supply rate of the substrates. As long as both glucose and methanol were completely consumed, the specific methanol uptake rate increased along with the dilution rate (Fig. 2, phases I and II), reaching its maximum of (0.090 ± 0.003) g\text{methanol} g\text{-1} DW h\text{-1} at the transition rate Dt. A further increase in the dilution rate caused a rapid decline in the specific methanol consumption rate to (0.044 ± 0.002) g\text{methanol} g\text{-1} DW h\text{-1} (Fig. 2, phase III), which was accompanied by an accumulation of part of the non-consumed methanol in the culture broth (Fig. 3, phase III). In contrast, glucose was completely utilized in all continuous cultures examined. The specific glucose and carbon uptake rates increased within the range of measured dilution rates, reaching maximum values of (0.251 ± 0.007) g\text{glucose} g\text{-1} DW h\text{-1} and (0.117 ± 0.004) g\text{carbon} g\text{-1} DW h\text{-1} respectively at a dilution rate of 0.20 h\text{-1} (Fig. 2). Accumulation of part of the non-consumed carbon originating from methanol caused a decrease of about 30% in the steady-state biomass concentrations achieved in cultures operated above Dt (Fig. 3).

3.3. Kinetics of product formation and cell viability

In continuous cultures, both productivity and the concentration of extracellularly produced porcine trypsinogen were highly dependent on the dilution rate. Stable formation of extracellular product was observed only in cultures grown at the low dilution rates of 0.03 h\text{-1} and 0.07 h\text{-1} (Fig. 4). The presence of recombinant trypsinogen in the culture broth was detected within two hours of induction and its concentration gradually increased to reach steady-state values of (210 ± 8) mg l\text{-1} and (157 ± 12) mg l\text{-1} at dilution rates of 0.03 h\text{-1} and 0.07 h\text{-1} respectively (Fig. 3). At the 0.07 h\text{-1} dilution rate, the highest specific and volumetric production rates of extracellularly produced trypsinogen (calculated using Eqs. (4) and (5)) reached steady-state values of (0.70 ± 0.02) mg g\text{-1} DW h\text{-1} and (10.9 ± 0.3) mg l\text{-1} h\text{-1} respectively (Fig. 2).

In contrast, in cultures grown at dilution rates exceeding 0.07 h\text{-1}, the concentration of porcine trypsinogen in the culture broth increased only for a certain time period (approx. 5 generations at 0.13 h\text{-1} and 2 generations at 0.20 h\text{-1}) and afterwards began to decline. Nevertheless, by this time, biomass, glucose and methanol concentrations had already reached steady-state values (Fig. 4). The concentration of recombinant trypsinogen was always above the curve of theoretical product wash-out (dashed line in Fig. 4, Eq. (5)), which suggests that the ability to excrete recombinant trypsinogen was progressively reduced rather than suddenly lost. Had the latter occurred, the concentration in the bioreactor would only have been diluted following the theoretical wash-out curve.

Fluorescent staining of *Pichia* cells with bis-(1,3-dibutylbarbituric acid) trimethine oxonol (BOX) and propidium iodide (PI), with subsequent analysis by flow cytometry, showed that the number of cells with disrupted cytoplasmic or depolarized membranes remained very low (~2% and 3% respectively) even if a decline in the production of trypsinogen occurred. An increase of less than 8% in both the percentage of PI-stained cells and BOX-stained cells was observed only in cultures with accumulated methanol (Fig. 3), regardless of whether the production of extracellularly secreted trypsinogen was stable or not. Thus, neither clear dissipation of the membrane potential nor disruption of the cytoplasmic membrane were observed by staining with BOX and PI respectively.

4. Discussion

The partial substitution of methanol with glucose was investigated as a cheap, readily available alternate carbon substrate, which is more appropriate for industrial scale-up than sorbitol, trehalose, mannitol, lactic or acetic acid (Celik et al., 2010; Inan and Meagher, 2001). To date, glucose has only been sporadically mentioned as a suitable substrate for the cultivation of *P. pastoris* Mut+ strains (Brierley et al., 1990; Hang et al., 2008; Okabayashi et al., 1997) and thus, no systematic data on carbon-limited continuous cultures have been published. Therefore, the data collected were compared to studies on the microbial physiology of AOX1-strains with methanol and glycerol mixtures (d’Anjou and Daugulis, 2001; Jungo et al., 2006; Ruth and Glieder, 2010; Sola et al., 2007; Wei et al., 2008; Zhang et al., 2003) as well as with pure methanol (Curvers et al., 2001; Issay et al., 2001; Yamawaki et al., 2007; Zhang et al., 2000, 2001).

In methylotrophic yeasts, the metabolism of methanol is typically regulated by repression, derepression and induction mechanisms, where glucose is referred to as one of the substrates that acts as a repressor of the methanol utilization pathway. Glucose will therefore inhibit induction of the AOX1 promoter in recombinant *P. pastoris* (Cos et al., 2006; Hartner and Glieder, 2006; Nakano et al., 2006; Sola et al., 2007). However, similar to the results of experiments performed with wild type *H. polymorpha* (Egli et al., 1986, 1982a) our data showed that it is possible to fully eliminate the repressive effect of glucose on both methanol utilization and product formation in continuous cultures (chemostat), provided such cultures are operated below the particular transition rate Dt. At this dilution rate, repression by glucose progressively sets in, methanol (but not glucose) accumulates in the medium, and biomass yield decreases correspondingly. For the strain producing recombinant porcine trypsinogen in the presence of a glucose and methanol mixture (60%-40% carbon), the Dt of 0.15 h\text{-1} was in a comparable range to the rates determined during the production of a recombinant lipase with a glycerol and methanol mixture (Sola et al., 2007) for mixtures with 20–60% carbon from methanol. The consumption of glucose by the AOX1 strain increased proportionally to the rising specific growth rate (i.e. dilution rate) up to the maximum of 0.25 g\text{glucose} g\text{-1} DW h\text{-1}.
reached at a dilution rate of 0.20 h\(^{-1}\), which is below the maximum value of 0.35 g\(_{\text{glucose}}\) g\(_{\text{CDW}}\) \(^{-1}\) h\(^{-1}\) determined at 0.193 h\(^{-1}\) for another Pichia construct producing an antibody fragment under the control of a different constitutive, non-regulated, non-inducible promoter (GAP), and thus grown in a culture with only glucose (Maurer et al., 2006).

Although glucose is commonly considered to be a strong repressor of the AOX1 promoter, and thus the utilization of glucose might hinder recombinant product formation, porcine trypsinogen was synthesized over the entire range of dilution rates studied up to 0.20 h\(^{-1}\). At the two lowest rates (0.03 h\(^{-1}\) and 0.07 h\(^{-1}\) ) the extracellular trypsinogen concentration, and therefore also productivity, was stable over the duration of the 4–6 generations investigated and was enhanced with increasing dilution rate. However at higher rates it was found to decrease as the dilution rate increased. In the literature, either growth-associated product formation patterns (e.g. Curvers et al., 2001; d’Anjou and Daugulis, 2001; Jungo et al., 2006) or the opposite, product formation patterns, which are not directly proportional to biomass growth and where production decreases with increasing specific growth rate (e.g. Issaly et al., 2001; Yamawaki et al., 2007), have been reported in respect of P. pastoris.

Although reduced productivity at rates above 0.07 h\(^{-1}\) was observed, product concentration was always above the curve of theoretical product wash-out (dashed line in Fig. 4, Eq. (7)), which suggests that the ability to excrete recombinant trypsinogen was
progressively reduced rather than suddenly lost. Consequently, the degradation of extracellularly secreted product due to both proteolytic degradation and autocatalytic activation of the protein has been reported as a unique feature of the trypsinogen system in high cell density fed-batch cultures (Hyka et al., 2010; Jahic et al., 2003a; Werten and de Wolf, 2005) but is not a cause of reduced productivity here. In all experiments carried out in this study, the product always occurred in the form of the inactive zymogen, and neither trypsin-like activity nor any products of trypsinogen self-digestion were determined in the samples taken from the bioreactors. In addition, neither the accumulation of methanol (Cunha et al., 2004; Curvers et al., 2001; Schenck et al., 2008; Zhang et al., 2000) nor the loss of the foreign gene through long-term cultivation (Curvers et al., 2001) was found to be the likely cause of declining productivity.

Neither the data on trypsinogen collected in this study (Fig. 2) nor the data published for different P. pastoris strains secreting a recombinant protein allow general conclusions to be drawn regarding the relationship between product formation and biomass growth. The complex mechanism of recombinant protein production might vary from system to system. It depends not only on the cultivation conditions (e.g. pH, temperature, oxygen availability) and physiology of the cells, but also the characteristics of a particular construct and the target product. In addition, the presence (or absence) of inducer/repressor molecules and precursors for product synthesis as well as the ease and accuracy of folding or of other post-translational modifications are reported to be important factors (Graf et al., 2009; Mattanovich et al., 2009b). Although this study highlights the benefits and suitability of substitution of part of the methanol with glucose, the regulatory mechanism at the gene level and the distribution of metabolic fluxes in cultures with a mixed substrate remain generally unknown. Certain information could be derived from comprehensive metabolic flux analysis studies of an AOX1-strain grown with different ratios of methanol/glycerol (Sola et al., 2007) as well as of a GAP-strain grown with glucose alone (Heyland et al., 2010). For instance, in comparison with the wild type strain, a more than 40% higher TCA-cycle flux was determined with a GAP-strain producing about 6 g l−1 of recombinant β-aminopeptidase at biomass concentrations higher than 200 g l−1, implying that this significantly increased flux stimulated protein synthesis (Heyland et al., 2010). Moreover, further metabolic studies along with transcriptomics based on microarray technology (Gasser et al., 2007; Graf et al., 2008; Mattanovich et al., 2008a), which are being advanced due to the current availability of the P. pastoris genome sequence (De Schutter et al., 2009), might deliver further insight into mixed substrate utilization in the near future. However, as illustrated here, straightforward investigations supported by monitoring methods that are also suitable for procedures at the industrial scale provide valuable physiological information. In contrast to high cell density cultures (>80 g l−1 CDW) where reduced viability of P. pastoris during production of recombinant proteins has been reported (Hohenblum et al., 2003, 2004; Hyka et al., 2010; Mattanovich et al., 2004), viabilities in chemostat culture (enumerated using flow cytometry method) were not significantly compromised. Even in cultures with accumulated methanol, the proportion of non-affected cells did not fall below 90%. This tendency towards damage of cells that were grown in continuous culture mode might be primarily due to the character of this cultivation system (Hewitt et al., 1999; Lu Chau et al., 2001), which allows fresh media to enter and metabolites as well as old cells to be washed out (in contrast to fed-batch). Thus, the negative effects which typically occur in batch or fed-batch cultures (for example, increasing stress associated with the presence of aged cells, proteases released from damaged cells, accumulation of products and metabolic by-products) are minimized.

5. Conclusions

Glucose was found to be a feasible partial substitute for methanol substrate in continuous cultures with recombinant P. pastoris secreting porcine trypsinogen under control of the AOX1 promoter (as also shown recently in high cell density fed-batch processes by Abdal et al. (2010); and Hyka et al. (2010)). The represive effect of excess concentrations of glucose on the metabolism of methanol and recombinant protein production observed in batch cultures with a mixture of methanol and glucose was entirely eliminated in continuous cultures controlled (i.e. limited) by the availability of both carbon substrates. Both glucose and methanol were completely and simultaneously consumed as long as the transition dilution rate D was not exceeded, which occurred approximately 30% higher than the maximum specific growth rate with methanol as the sole substrate (0.116 h−1). It can therefore be concluded that (1) higher biomass productivities are, in principal, achievable with a substrate mixture rather than with methanol alone; (2) that the use of glucose as a carbon and energy source might directly contribute to enhancing the performance of protein production in P. pastoris. Productivity is enhanced particularly due to the considerably higher yield of biomass per consumed substrate, higher protein production rates, lower heat evolution and a high proportion of actively producing cells.

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